PRENATAL DIAGNOSIS
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PRENATAL DIAGNOSIS FOR THALASSAEMIA IN A MULTICULTURAL SOCIETY

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SUMMARY

The provision of a prenatal diagnosis service for thalassaemia is becoming more demanding. In an ethnically-diverse community, the number of mutations has increased. Requests for prenatal testing continue to come at an advanced stage in pregnancy, often without the underlying mutation having been identified. Although controls are included in PCR assays, errors can still occur. The alternative to DNA testing, i.e., an α/β globin chain synthesis ratio on a fetal blood sample, is now less readily available. In the circumstances described, the laboratory must adopt a more efficient and reliable approach to DNA mutation analysis. With currently available technology, this improvement is more likely to come through increased automation. To achieve this aim, we have moved to capillary electrophoresis. With capillary electrophoresis we are able to use a PCR-based *screening* strategy which can detect up to 11 β thalassaemia mutations. The *actual* prenatal test is undertaken using two independent PCRs thereby reducing the potential for error. Despite the advantages of PCR, $\sim$12 per cent of β thalassaemia and about nine per cent of α thalassaemia cases require further study in our experience. In this situation, capillary electrophoresis has again proven helpful since a DNA *scanning* approach, such as single strand conformation polymorphism, can be automated to identify the region of DNA to be sequenced. © 1998 John Wiley & Sons, Ltd.

KEY WORDS: thalassaemia; DNA; prenatal diagnosis; capillary electrophoresis

INTRODUCTION

The haemoglobinopathies illustrate the various developments which have occurred in the prenatal diagnosis of genetic defects. The first case of fetal blood sampling to detect β thalassaemia by measurement of the protein's α/β globin chain ratio was reported over 20 years ago (Kan *et al.*, 1975). This was followed by DNA testing of amniocytes for the sickle cell defect (Chang and Kan, 1982). Another milestone was the use of a chorionic villus sample as the DNA source. Now, prenatal testing could be conducted during the first trimester of pregnancy (Williamson *et al.*, 1981). From the mid-1980s, DNA analysis gradually replaced the traditional fetal blood sampling approach. DNA strategies included indirect linkage analysis, which was required because of the large number of point mutations found in the β thalassaemias, and DNA gene mapping to detect the deletions found in the α thalassaemias. Mutation detection by allele specific oligonucleotide (ASO) hybridization for individual point mutation in the β thalassaemias also became possible (Orkin, 1984).

Although providing an alternative to fetal blood sampling, the necessity to use the traditional Southern blotting approach for DNA gene mapping meant that it took a minimum of two weeks

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to obtain a diagnosis. ASO hybridization using total genomic DNA required considerable technical skills. Unlike fetal blood sampling, DNA testing could not be undertaken in all pregnancies, e.g., there was no index case essential for linkage analysis, or couples presented too late and so there was insufficient time for the available DNA techniques to provide an answer. In these circumstances, the second, but less acceptable choice, because it involved second trimester diagnosis, remained an α/β globin chain ratio from a fetal blood sample.

The polymerase chain reaction (PCR) was described in 1985. PCR facilitated the detection of single point mutations and greatly expanded the range of β thalassaemia defects which could be detected (Old *et al.*, 1990). The necessity to have an index case for a linkage study was removed. Thus, an increasing number of couples were able to request DNA-based prenatal diagnosis during their first pregnancy. PCR could also be used to detect deletions in the α and $(\delta\beta)^0$ thalassaemias (Bowden *et al.*, 1992; Craig *et al.*, 1994). The potential to obtain a PCR result within a shorter time frame than was possible with Southern blotting also meant that couples who presented late, were still able to undergo prenatal testing, with the major constraint in this situation being the legal time limit for termination of pregnancy.

In the late 1990s, a new trend has emerged in communities with a changing ethnic background. Australian cities, such as Sydney, have experienced both an increase in population as well as a shift in the country of birth for new settlers. In Sydney, the traditional southern European link associated with thalassaemia (especially Greece, Cyprus and Italy) has broadened to include immigrants coming from different at-risk geographic regions, such as China, South East Asia, India and the Middle East. For example, in 1995–96, new arrivals to Australia originated from: China (11·3 per cent), Hong Kong (3·7 per cent), India (3·6 per cent), Vietnam (3·4 per cent), Bosnia-Herzegovinia (3·4 per cent), Phillipines (3·3 per cent) (Australian Department of Immigration and Multicultural Affairs Fact Sheet 33, 1997: http://www.immi.gov.au/facts/33STATS.htm). Since many of these communities have specific thalassaemia mutations associated with them (Thein, 1993), the scope for mutation detection has had to be expanded.

At the same time as the DNA developments described earlier have evolved, the option for fetal blood sampling has diminished because of the falling demands which occurred in the early 1990s. Even laboratories that were proficient in obtaining an α/β ratio in a fetal blood sample are now finding that the infrequent demand on this service is insufficient to ensure quality of results. Despite greater public awareness and education programmes directed at antenatal care, the number of at-risk couples presenting late continues, and may even be increasing. In some cases, this can be attributed to cultural or religious beliefs. Alternatively, there is a lack of knowledge of antenatal facilities often as a result of communication problems. In this environment, it has become necessary to improve the efficiency and accuracy of DNA mutation detection to take into account an expanded range of mutations as well as late presentations. One way in which this can be done is to increase the scope for automation. This study describes the utility of capillary electrophoresis as a means by which DNA prenatal testing for the thalassaemia syndromes can be further developed.

MATERIALS AND METHODS

Patients

Data on ethnic backgrounds, the range of mutations and outcomes were obtained by summarizing the experience of the NSW prenatal DNA testing service from the years 1984 to early 1997. This laboratory serves a population of approximately 6·1 million. It also receives some referrals from the state of Queensland and New Zealand.

DNA mutation analysis

Prior to 1997, PCR approaches used for β thalassaemia DNA mutation analysis included ASO hybridization (as individual mutations and also as a multiplex reaction) and alterations in the restriction enzyme patterns produced by the underlying defect. When mutations could not be detected, linkage analysis was attempted if an appropriate family structure was available. When the above failed, the option of DNA sequencing or fetal blood sampling was considered. Mutation analysis for the South East Asian and Mediterranean types of α^0 thalassaemia was undertaken by PCR (Bowden *et al.*, 1992).

Capillary electrophoresis

During 1997, the approach to DNA mutation analysis was changed into a three-step process:

Table I—β globin gene mutations detected

Mediterranean-specific mutation	Number	Per cent	Chinese/Asian, Indian-specific mutation	Number	Per cent
*IVS1-110 (G→A)	108	53	*-28 (A→G)	10	28
*IVS1-6 (T→C)	32	16	*CD41/42 (-TCTT)	9	26
*CD39 (C→T)	22	11	IVS2-654 (C→T)	8	22
*IVS1-1 (G→A)	21	10	*IVS1-5 (G→C)	4	11
IVS2-745 (C→G)	12	6	*IVS1-1 (G→T)	1	3
*IVS2-1 (G→A)	4	2	*CD17 (A→T)	1	3
CD5 (-CT)	3	2	*CD30 (G→C)	1	3
-87 (C→G)	1	<1	ATG→AGG	1	3
			CD71/72 (+A)	1	3

*Form part of the multiplex; CD—codon; IVS—intervening sequence; %—the percentage of mutations obtained from the total number of thalassaemia chromosomes studied.

screening for known mutations, the actual prenatal test and DNA scanning by SSCP (single strand conformation polymorphism) for less common or novel mutations. Increased flexibility became possible because different coloured fluorescent-labelled oligonucleotide primers were used in an ARMS-based methodology (ARMS—amplification refractory mutation system) (Newton *et al.*, 1989; Old, 1996a). Fluorescent dyes included 6-FAM (blue), TET (green), HEX (yellow) and TAMRA (red). The latter was reserved for DNA size markers which could then be added to the test samples prior to electrophoresis. PCR products were able to be separated and their sizes measured by an automated capillary electrophoresis approach using an ABI PRISM 310 Genetic Analyser (Perkin Elmer, Foster City).

(i) Screening of parental DNA samples for known β thalassaemia mutations was undertaken with one of three multiplex capillary electrophoresis–ARMS assays which incorporated primers for mutant alleles alone. Multiplex 1—Mediterranean mutations: codon (CD) 39, IVS1-1 (G→A), IVS1-110, IVS1-6 and IVS2-1. Multiplex 2—predominantly Chinese/Asian, Indian mutations: -28, CD17, CD30, IVS1-1 (G→T), IVS1-5 and the CD41/42 deletion. Multiplex 3—a combination of multiplex 1 and 2, i.e., 11 mutations which were looked for in cases of unknown or mixed ethnic backgrounds. If these were negative, additional mutations, e.g., Mediterranean: IVS2-745, CD5, -87; Chinese/Indian Asian: IVS2-654 and CD71-71 were sought individually or DNA sequencing considered (see (iii) below) (Table I). Each of the three multiplexes involved a single PCR step and one capillary electrophoresis run. Primer sequences, PCR conditions are available on request.

(ii) For the actual prenatal study, two different PCRs were used i.e., a mutation-specific ARMS test using both normal and mutant primers and a separate confirmatory ASO hybridization or restriction enzyme pattern analysis.

(iii) DNA scanning by SSCP was used to facilitate sequencing by identifying regions of DNA likely to contain single base changes (Orita *et al.*, 1989). This involved the amplification of individual β globin gene exons using fluorescent-labelled primers. Single-stranded DNA fragments were then separated by capillary electrophoresis under non-denaturing conditions and a temperature of 29°C. Fragments undergoing a mobility shift were sequenced. Primers, PCR and electrophoresis conditions are available on request. An added benefit of the ABI PRISM 310 Genetic Analyser was that this instrument could also be used for automated DNA sequencing.

RESULTS

Thalassaemia profile

Over a period of 14 years (1984–1997) there have been 255 prenatal DNA tests undertaken for thalassaemia (58 for α thalassaemia and 197 for β thalassaemia). Ethnic backgrounds for at-risk

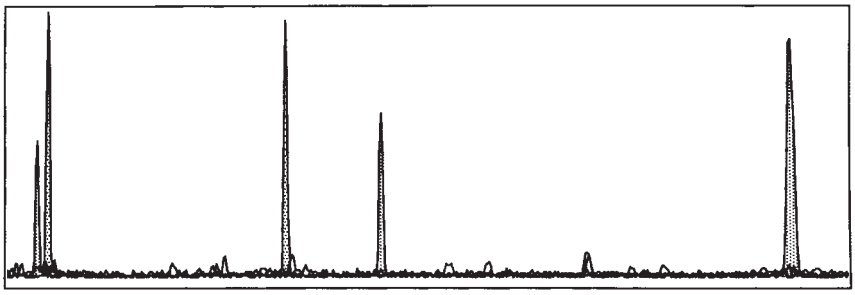

Fig. 1—Multiplex 1—from left to right the mutations are IVS1-1 (G→A), IVS1-6, IVS1-110, CD39 and IVS2-1. The horizontal axis represents the time taken for the DNA to migrate through the capillary. The vertical axis is the signal intensity calculated with a sensitive CCD camera sensor. This provides an additional check point, since the intensities of bands provide some information of a semi-quantitative nature. Thus, compared with appropriate controls, the peak for a homozygous-affected or normal sample would be expected to be significantly higher than one from a heterozygote

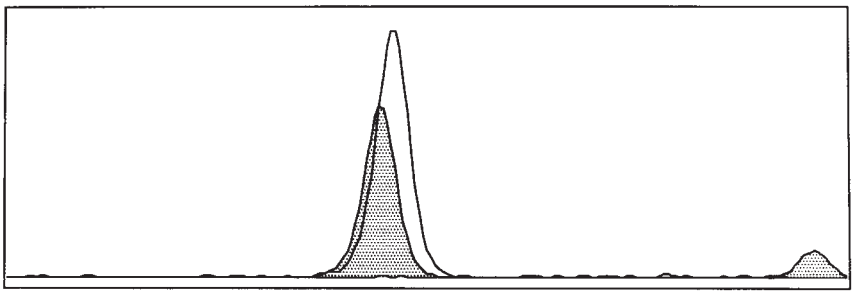

Fig. 2—Multicolour-labelled primers. As well as avoiding the use of slab gels in capillary electrophoresis, the advantage of multicolour fluorescence is that samples which co-migrate are able to be distinguished. In this particular case, the mutant and normal alleles for the IVS1-110 mutation have been labelled with different fluorochromes, and each can be detected in the one PCR and electrophoresis step. The small peak to the right is a size marker

individuals were: Mediterranean (57 per cent); Chinese/South East Asian/Indian (33 per cent); Middle Eastern (seven per cent) and others (three per cent). The range of mutations tested is summarized in Table I.

DNA mutation screening

The capillary electrophoresis–ARMS-based multiplex screening strategy enabled over 90 per cent of the Mediterranean-type β thalassaemia mutations to be detected and approximately 74 per cent of those of Chinese/Asian, Indian origin (Table I, Fig. 1). This approach was considerably faster than testing for individual defects and, once standardized, more reproducible and technically easier than multiplex ASO hybridization. The advantage of multicolour fluorescence was quickly apparent since PCR fragments of similar size were able to be distinguished on the basis of colour (Fig. 2). Expanding the number of mutations would be possible by selecting appropriate primers and PCR conditions.

Mutations in the second β globin gene intron were not included in the multiplex because they are located at some distance from the majority of defects which cluster in exon 1. This clustering of mutations can, in itself, make it difficult to design a suitable multiplex using traditional slab gels which require about 10–20 base pair differences for adequate separation. In this respect, capillary electrophoresis is more flexible, since it can adequately resolve fragments which differ by 1–2 base pairs.

Prenatal DNA test

In the period from 1990–1996, which was selected because the technology for individual

mutation detection was in full use, 18 of the 148 β thalassaemia studies (12 per cent) and four of the 44 α thalassaemia cases (nine per cent) were complicated by the fact that only one or neither of the two mutations was detectable in each couple. In some cases the only known mutation was not present in fetal DNA, and so the prenatal test was informative in that it excluded a homozygous-affected fetus. However, this may not be the outcome in subsequent pregnancies. DNA polymorphisms were informative in six of the 18 β thalassaemia studies. In two cases sufficient notice was given to the laboratory for DNA sequencing to identify an underlying mutation (-87 C→G in a Greek male and the CD30 AGG→ACG mutation in a couple who came from the Maldives). DNA sequencing was time consuming, since it was undertaken blindly. Nine (seven β, two α) prenatals required fetal blood sampling for a definitive result, i.e., the demand on the laboratory for this test was about one to two per year.

There was one recognized PCR-based error in the 255 prenatal tests. This occurred in a couple with mixed ethnic background (mother Chinese, father Middle Eastern) at risk of β thalassaemia. A CD41/42 mutation (deletion of four bases-TCTT) was detected in the maternal DNA sample at the time of the prenatal test but not in the chorionic villus sample, which was shown to have the paternal IVS1-110 mutation. Following birth of the child and subsequent development of severe anaemia, the original fetal DNA sample was retested ~15 months later using different reagents. A weak signal consistent with the CD41/42 deletion was now evident (Fig. 3).

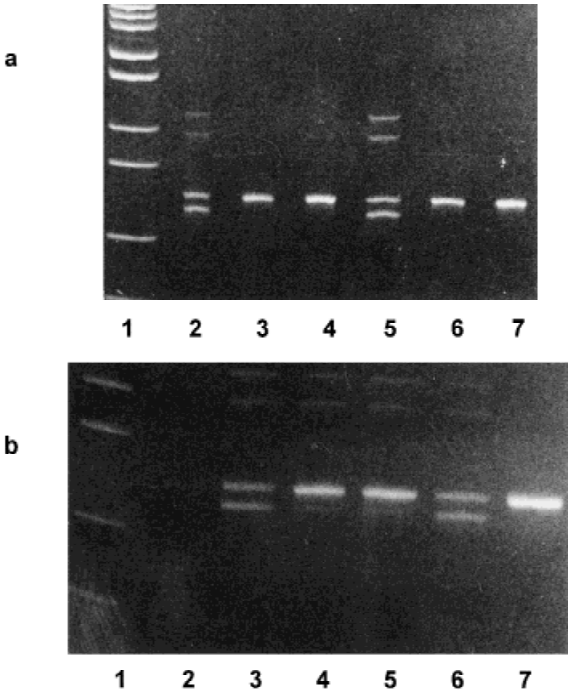

Fig. 3—Differential amplification of normal and mutant alleles. The four base pair deletion associated with the CD41/42 mutation is easily detectable by a PCR which shows, in the heterozygote, two bands representing the normal allele and the faster migrating mutant allele with an additional heteroduplex above them in the case of a heterozygote. (a) 1—Size marker, 2—maternal DNA, 3—paternal DNA, 4—fetal DNA, 5—positive control for CD41/42 mutation, 6—normal DNA control, 7—fetal DNA. The no DNA control track is not shown. (b) Repeat assay ~15 months later. 1—size marker, 2—no DNA control, 3—maternal DNA, 4, 5—fetal DNA (note the feint mutant band but a visible heteroduplex), 6—positive control for CD41/42, 7—normal DNA control

DNA scanning (SSCP)

In the first two individuals studied by capillary electrophoresis-SSCP (a β thalassaemia homozygous-affected Lebanese child and an unrelated carrier parent of Chinese background), the exon 1 SSCP was suspicious since it showed either a mobility shift in the band or a double peak (Fig. 4). Direct DNA sequencing of exon 1 confirmed that the affected child was homozygous for the CD5 (-CT) mutation and the carrier parent had the ATG to AGG start codon defect.

DISCUSSION

What can be achieved in a carrier screening and prenatal diagnosis programme to reduce the frequency of homozygous β thalassaemia is illustrated by the results which have come from Sardinia (Cao, 1994). In this population of approximately 1·5 million people, the newborn birth rate for homozygous β thalassaemia has been reduced from one in 250 to one in 4000. However, the population is relatively homogeneous, and two defects (CD39 and CD6 (-A)) account for nearly 98 per cent of the mutations. Educational programmes for the population and health providers could be mounted within the context of a common ethnic background. The other end of the spectrum is to be found in a city such as Sydney, where the at-risk groups have originated from many continents, and so the range of thalassaemia mutations is considerable, and continues to change. In this environment, the different customs

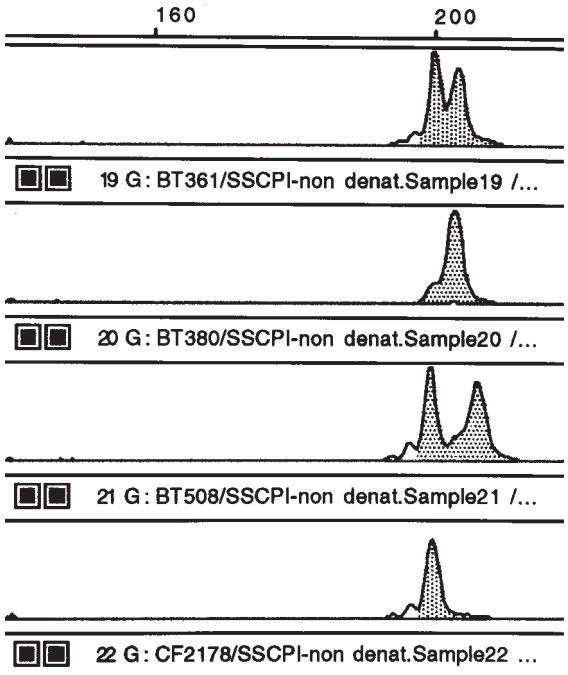

Fig. 4—Automated SSCP using capillary electrophoresis. Sample 19 is a known heterozygote for the IVS1-1 (G→T) mutation. Sizes of the two peaks relative to the internal DNA standards are 200·00 bp (bp—base pairs) and 203·60 bp; sample 20 is the unknown homozygous affected child (one peak which is 203·21 bp in size); sample 21 is the unknown adult carrier (peaks at 199·77 bp and 206·44 bp); sample 22 is the normal control which shows a single peak at 200·00 bp. There is a suspicious SSCP pattern for unknown samples 20 and 21. The figure also illustrates the accuracy obtained with automated fragment sizing (normal = 200 bp) which would not be possible by conventional slab gel electrophoresis and visual size estimation

and communication problems complicate the educational prerequisites for screening. This places additional pressure on the DNA diagnostic laboratory because of late presentations, and a lack of knowledge of the mutations which will need to be detected. Community groups, especially the NSW Thalassaemia Society, provide an outstanding service in terms of education and support, but the involved parties are predominantly of Mediterranean origin and there is little interaction with the Chinese/Asian, Indian and Middle Eastern communities.

The laboratory which carries out a very limited number of α/β biosynthesis studies, a method which is accurate and not constrained by the necessity to detect a mutation, is either stopping this service because the throughput is insufficient to maintain the skills of the operators, or results are provided with considerable trepidation. With the constraints described, DNA mutation analysis must become more flexible and efficient, and, at the same time, the standards or protocols adopted less error-prone. Traditional methods to screen and detect single base changes usually found in the β thalassaemias include techniques such as ARMS or hybridization approaches which include ASO and reverse dot blotting. These techniques can be multiplexed, but still require the preparation of gels or the blotting of DNA onto filters, staining or autoradiography and then interpretation of data. A major limitation to multiplexing in the traditional ARMS test is the difficulty in distinguishing co-migrating bands and so individual samples for the mutant and normal alleles may need to be electrophoresed separately. We have approached the changes which are occurring, and the difficulties described, by increasing the utilization of automated technology.

Capillary electrophoresis is a rapidly growing technique which allows DNA (and other) fragments to be separated on the basis of size or charge following electrophoresis in a capillary (Altria, 1996; Barron and Blanch, 1995). The ability to dissipate heat from a capillary enables high voltages to be generated, which increases the efficiency and resolving capacity of this technology. Compared with the conventional slab gel electrophoresis, capillary electrophoresis is automated. A minute amount of sample is required for analysis, e.g., 10 nl, and the separated product is visualized by a very sensitive electronic detector which displays the results on a screen prior to a report being printed. If necessary, an unsatisfactory sample can be programmed for repeating while a batch of specimens are still being analysed. A number of capillary electrophoresis platforms are now in the market-place. Capillary electrophoresis, which is enhanced by the ability to label primers with different fluorochromes, is available in the ABI 310 Genetic Analyser. The potential to utilize different colours allows co-migrating DNA fragments to be distinguished. This increases further the flexibility of the technique in terms of its ability to expand the range of mutations which can be detected in the one multiplex. As illustrated in the present study, a capillary electrophoresis–ARMS multiplex enabled 11 β thalassaemia mutations to be screened for in a single step. We are presently attempting to multiplex the SSCP assay so that it

will become possible to make greater use of DNA sequencing in problem cases. It is anticipated that multiplexing will be considerably easier to achieve by the availability of different coloured primers, since the location of any aberrant band will be identifiable by its colour.

The detection of α thalassaemia deletions is considered to be more accurately undertaken with the traditional Southern blotting approach (Old, 1996b). However, in laboratories which provide a DNA diagnostic service for a range of disorders, e.g., thalassaemia, cystic fibrosis, Huntington's disease, the overwhelming requirement for PCR has meant that staff are now less experienced in Southern blotting and so must rely on PCR-based methods, even if a particular PCR, such as that for α thalassaemia, is technically demanding. A number of precautions are recommended to reduce the likelihood of error when using PCR for prenatal diagnosis. These include inspection of the chorionic villus sample and removal of decidual tissue, limiting the number of amplification cycles, analysing in duplicate with different molecular approaches and using microsatellites to monitor for maternal contamination (Old, 1996b). The capillary electrophoresis–multicoloured ARMS approach described facilitates the latter two options, thereby allowing more time for a second and unrelated PCR test to confirm the result. The sensitivity possible with capillary electrophoresis ensures that partial amplification products, if these occur because of limitations in the PCR, are more likely to be detected. The reproducibility obtainable with automated fragment sizing provides the laboratory with an additional safeguard in terms of quality assurance.

The mutation model described for thalassaemia would apply equally to other genetic disorders. For example, in the multicultural society described in this particular study, a diagnostic difficulty is occurring with cystic fibrosis in the Lebanese community since the ΔF508 and other more common mutations are less frequently detected. The increasing availability of DNA diagnostic kits will expand the range of mutations detected. Kits which rely on a change in a DNA fragment size as the basis for mutation analysis can be used in conjunction with capillary electrophoresis. In future, the potential for automation will be further expanded when it becomes possible to include a robotics workstation to perform the PCR step, and from this, automatically load amplified products for capillary electrophoresis.

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